BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

APPELLANTS' BRIEF Address to: Box AF Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Attorney Docket	UCAL-269
	First Named Inventor	Prabhakara V. Choudary
	Application Number	09/425,075
	Filing Date	October 21, 1999
	Group Art Unit	1642
	Examiner Name	Larry Ronald Helms
	Title	*Functionally assembled antigen-specific intact recombinant antibody and a method for production thereof*

Sir:

This Brief is filed in support of Appellants' appeal from the Examiner's Final Rejection dated September 12, 2003. No claims have been allowed, and claims 36-39 and 41-50 are pending. All claims are appealed. A Notice of Appeal was filed on December 12, 2003.

The Board of Appeals and Interferences has jurisdiction over this appeal pursuant to 35 U.S.C. §134.

The Commissioner is authorized to charge to Deposit Account No. 50-0815 the required fee of $310.00 to cover the $165.00 required per 37 C.F.R. §1.17(c) for filing appellants' brief and the $145.00 for the Request for Oral Hearing. In the unlikely event that the transmittal papers are separated from this document and/or other fees or relief are required, appellants petition for such relief, including extensions of time, and authorize the Commissioner to charge any fees under 37 C.F.R. §§ 1.16, 1.17 and 1.21 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number UCAL-269.

This Appeal Brief, and any appendices, exhibits and references accompanying this brief, are submitted in triplicate.

TABLE OF CONTENTS

REAL PARTY IN INTEREST

The real party of interest in this appeal are The Regents of the University of California, who is the assignee.

RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

STATUS OF THE CLAIMS

This application claims the benefit of U.S. provisional patent application serial number 60/105,259, filed October 22, 1998.

Claims 1-35 and 40 were cancelled during prosecution.

All pending claims (claims 36-39 and 41-50) are rejected and are appealed.

The appealed claims are listed in Appendix I.

STATUS OF AMENDMENTS

All amendments to the claims have been entered.

SUMMARY OF THE INVENTION

Appellants claim a method for producing an antibody in the methylotrophic yeast *Pichia* using a "dual expression cassette vector" (i.e., a vector containing two expression cassettes, one for expressing each of the two chains of an antibody (heavy chain and light chain)). (see, e.g., page 6, line 32 to page 7, line 7; page 9, line 9 to page 10, line 12; page 24, line 27 to page 25, line 17; page 27, lines 5-27; (Example 2) and Figure 1 (description at page 4, line 32 to page 5, line 4); and Figure 6 (showing detecting of secreted antibody)). According to the claimed methods, a *Pichia* cell containing such a dual expression cassette vector is cultured to provide for expression of antibody light and heavy chains, and the antibody is harvested from culture supernatant. (page 7 lines 3-7).

Appellants also claim a *Pichia* expression vector that is a "dual expression cassette" vector having two expression cassettes which comprise nucleic acid encoding the heavy and light chains of an antibody, where the vector provides for production of the antibody in a *Pichia* host

cell (see, e.g., page 6, line 32 to page 7, line 7; page 9, line 9 to page 10, line 12; page 27, lines 5-27; (Example 2) and Figure 1 (description at page 4, line 32 to page 5, line 4)).

Appellants also claim a recombinant *Pichia* cell containing such an expression vector (page 6, line 32 to page 7, line 7; page 10, line 26 to page 11, line 21; page 24, line 27 to page 25, line 17; page 29, line 20 to page 30, line 11).

Claims 36, 47 and 48 are illustrative of the method, vector, and recombinant cell claims on appeal, respectively:

> 36. A method for production of an antibody that specifically binds an antigen of interest, the method comprising the steps of:
>
> culturing a recombinant *Pichia* cell, the cell comprising a vector comprising a first and a second expression cassette, wherein:
>
> said first expression cassette comprises a first promoter operably linked to a nucleic acid encoding an immunoglobulin light chain operably linked to a first signal peptide;
>
> said second expression cassette comprises a second promoter operably linked to a nucleic acid encoding an immunoglobulin heavy chain operably linked to a second signal peptide,
>
> and said culturing provides for expression of the immunoglobulin light and heavy chains; and
>
> harvesting specific antigen-binding antibody from culture supernatant, which antibody specifically binds an antigen of interest.

47. A *Pichia* expression vector comprising:

a first and a second expression cassette, said first cassette comprising a first promoter operably linked to a nucleic acid encoding an immunoglobulin light chain operably linked to a first signal peptide, and said second cassette comprising a second promoter operably linked to a nucleic acid encoding an immunoglobulin heavy chain operably linked to a second signal peptide,

wherein introduction of said vector into a *Pichia* host cell provides for production of a recombinant immunoglobulin protein that specifically binds an antigen and is secreted by the host cell.

48. A recombinant *Pichia* cell containing the expression vector of claim 47.

Accordingly, a method for producing an antibody in *Pichia* using a dual expression cassette vector is being claimed. Also claimed is a dual expression cassette vector and recombinant *Pichia* cell as used in the subject methods.

The subject methods have general utility for production of large quantities of an antibody using the methylotrophic yeast, *Pichia pastoris*. (page 25, lines 19-21).

ISSUES

There are two issues on appeal, each relating to rejections of the pending claims for obviousness under 35 U.S.C. §103(a), as follows:

I. WHETHER THE INVENTION AS CLAIMED IN CLAIMS 36-39 AND 42-50 IS OBVIOUS UNDER 35 U.S.C. §103 IN VIEW OF HORWITZ, CREGG, THE INVITROGEN CATALOG AND ROBINSON

Claims 36-39 and 42-50 stand rejected in the Office Action of September 12, 2003, under 35 U.S.C. §103 as being unpatentable over Horwitz (PNAS 85:8678-8682, 1988) and further in view of Cregg (Developments in Industrial Microbiology 29:33-41, 1998); The Invitrogen

Catalog (1997) (published 1/97, Yeast expression pages 14-19 and Master Catalog Amendment Notice for pPICZ vectors form 4/15/96); and Robinson (USPN 6,204,023).

Horwitz is cited for its disclosure of a *single* expression cassette vector system for production of functional antibodies in *S. cerevisiae*. Cregg is cited for its disclosure of a *Pichia* alcohol oxidase promoter. The Invitrogen Catalog is cited for its disclosure of a *single* expression cassette vector system for use in *Pichia*. Robinson is cited for its disclosure of a *dual* expression cassette system for producing functional antibodies in mammalian cells, and for its asserted suggestion that such a dual expression cassette system could be used for antibody production in "yeast". The issue on appeal here is whether the rejected claims directed to a method of producing an antibody in *Pichia* using a *dual* expression cassette vector, as well as claims directed to such dual expression cassette vectors and recombinant *Pichia* cells containing such vectors, are obvious from these combined disclosures.

II. WHETHER THE INVENTION AS CLAIMED IN CLAIMS 36-39 AND 41-50 IS OBVIOUS UNDER 35 U.S.C. §103 IN VIEW OF HORWITZ, CREGG, THE INVITROGEN CATALOG, ROBINSON, AND VANDERLAAN

Claims 36-39 and 41-50 stand rejected under 35 U.S.C. §103 as being unpatentable over Horwitz (PNAS 85:8678-8682, 1988) and further in view of Cregg (Developments in Industrial Microbiology 29:33-41, 1998), The Invitrogen Catalog (1997) (published 1/97, Yeast expression pages 14-19 and Master Catalog Amendment Notice for pPICZ vectors form 4/15/96), Robinson (USPN 6,204,023), and Vanderlaan (USPN 5,429,925).

Horwitz is cited for its disclosure of a *single* expression cassette vector system for production of functional antibodies in *S. cerevisiae*. Cregg is cited for its disclosure of a *Pichia* alcohol oxidase promoter. The Invitrogen Catalog is cited for its disclosure of a *single* expression cassette vector system for use in *Pichia*. Robinson is cited for its disclosure of a *dual* expression cassette system for producing functional antibodies in mammalian cells, and for its asserted suggestion that such a dual expression cassette system could be used for antibody production in "yeast". Vanderlaan is cited for its disclosure of an anti-dioxin antibody. The issue on appeal is whether the rejected claims are unpatentable under 35 U.S.C. §103(a) in view of these combined disclosures.

GROUPING OF THE CLAIMS

As to each ground of rejection set out in the Office Action mailed September 12,2003, claims 36-39 and 41-50 are argued together, and as such stand or fall together with respect to each ground of rejection.

ARGUMENT

The two issues on appeal are each rejections of the claims under 35 U.S.C. §103(a) which are based on a combination of references – namely Horwitz, Cregg, The Invitrogen Catalog and Robinson (Issue I) and these same references further combined with Vanderlaan (Issue II). Thus, the arguments presented below with respect to Issue I apply with equal force to Issue II.

I. THE INVENTION AS CLAIMED IN CLAIMS 36-39 AND 42-50 IS NOT OBVIOUS UNDER 35 U.S.C. §103 IN VIEW OF HORWITZ, CREGG, THE INVITROGEN CATALOG AND ROBINSON

Claims 36-39 and 42-50 stand rejected as *prima facie* obvious in view of Horwitz, Cregg, The Invitrogen Catalog and Robinson as discussed above.

The M.P.E.P. provides clear guidance on the requirements of a *prima facie* case of obviousness:

> To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations." M.P.E.P. § 2142.

Thus, in order to render a claimed invention obvious, there must be some suggestion to modify or combine the cited references to provide the claimed invention. Therefore, in order to render the appealed claims obvious in view of the cited references, there must be a suggestion to use a ***dual* expression cassette vector** to express an antibody in ***Pichia*** (claims 36-39, 41-46 and 49-50, as well as dual expression cassette vectors (claim 47), and recombinant *Pichia* containing such dual expression cassette vectors (Claim 48).

Further, in order to render a claimed invention obvious, there must be a reasonable expectation of success in practicing the claimed invention. Therefore, in order to render the appealed claims obvious in view of the cited references, one of skill in the art must reasonably expect success in using a dual expression cassette vector to produce antibodies in *Pichia*, and in making such vectors and recombinant *Pichia* cells.

The Appellants respectfully submit that:

a) the rejection is in error because the Office has not shown that the cited art provides any motivation or suggestion to use a ***dual*** expression cassette vector **in *Pichia***, and

b) the rejection is in error because one of skill in the art would have no reasonable expectation of success in practicing a method of producing an antibody in *Pichia* using a dual expression cassette vector.

Accordingly, the Office has failed to establish a *prima face* case of obviousness, and this rejection should be withdrawn. Reasoning in support of the Appellants position is set forth below.

a) The cited references provide no motivation to make and use dual expression cassettes for antibody production in Pichia

The rejection is based upon a combination of references in order to provide all the elements of the claims. The disclosures of Horwitz, Cregg and the Invitrogen Catalog relied upon by the Office are as follows:

- Horwitz is cited for its disclosure of a ***single*** expression cassette vector system for production of functional antibodies in ***S. cerevisiae***.
- Cregg is cited for its disclosure of a *Pichia* alcohol oxidase promoter.
- The Invitrogen Catalog is cited for its disclosure of a ***single*** expression cassette vector system for use in ***Pichia***.

None of these references provide any disclosure relating to a dual expression cassette vector or the use of same in *Pichia* as required by the claims. In an erroneous attempt to cure this deficiency of these combined disclosures, the Office has relied upon Robinson.

Robinson is cited on the grounds that it provides a key element lacking in each of the other references -- namely a dual expression cassette vector. However, at no point does Robinson

suggest that a dual expression cassette **for use in *Pichia***, or, for that matter, **even mention the word "*Pichia*".** At best, Robinson suggests – but does not show - using a dual expression cassette vector in "yeast". In order to establish the "motivation" to combine the references as required under the law of obviousness, the Office has erroneously interpreted the word "yeast" as used in the context of Robinson to mean a genus of microorganisms that encompasses *Pichia*. The Appellants respectfully disagree with this interpretation of the word "yeast" in the context of Robinson, and respectfully submit that the disclosure of "yeast" in Robinson cannot be extended to encompass *Pichia*.

As is well established in the Courts, and as set forth in MPEP § 2123, a reference may be relied upon for all that it reasonably suggests to one having ordinary skill in the art.[1] Accordingly, a teaching of the reference can not be viewed in isolation, but rather must be considered in the context of what it would have suggested to one of skill in the art.

The Appellants respectfully submit that, to one of ordinary skill in the art, the word "yeast", as used in the context of Robinson, refers to a species of yeast that is commonly known as brewers' yeast, *S. cerevisiae*. The use of the word "yeast" in Robinson does not encompass the methylotrophic yeast *Pichia*. Accordingly Robinson provides ***no*** suggestion to use such a vector for antibody production in *Pichia*.

The Appellants' position is supported both from an analysis of the use of "yeast" in Robinson itself, as well as by evidence made of record in the form of a declaration under 37 C.F.R. §1.132.

First, Appellants point out that Robinson uses the terms "yeast" and "*S. cerevisiae*" interchangeably throughout the reference. For example, Robinson refers to the *S. cerevisiae* gene as "the yeast invertase gene", (Robinson, col.44, lines 46-47) refers to the *S. cerevisiae* PGK promoter as "the yeast PGK promoter", (Robinson, col. 9, line 50) and refers to the origin of

---

[1] MPEP § 2123 :A reference may be relied upon for all that it would have reasonably suggested to one having ordinary skill the art, including nonpreferred embodiments." citing *Merck & Co. v. Biocraft Laboratories*, 874 F.2d 804, 10 USPQ2d 1843 (Fed. Cir.), *cert. denied,* 493 U.S. 975 (1989). See also *Celeritas Technologies Ltd. v. Rockwell International Corp.*, 150 F.3d 1354, 1361, 47 USPQ2d 1516, 1522-23 (Fed. Cir. 1998) (The court held that the prior art anticipated the claims even though it taught away from the claimed invention. "The fact that a modem with a single carrier data signal is shown to be less than optimal does not vitiate the fact that it is

replication of the 2-micron plasmid endogenous to *S. cerevisiae as* "the yeast origin of replication, oriY, a cis-acting sequence (REP3) from the yeast endogenous 2-micron plasmid." (Robinson, col. 45, line 65-col 46, line 2). At no point in the disclosure does Robinson define "yeast" as anything other than *S. cerevisiae,* and never uses the term "yeast" to describe anything other than *S. cerevisiae*. Furthermore, the relevant examples in Robinson relating to antibody expression in "yeast" only describe use of *S. cerevisiae*. As such, one of skill in the art would recognize that "One preferred host is yeast" (column 15, line 39), as cited by the Examiner to establish the "motivation" aspect of this rejection, is directed *S. cerevisiae*, **not *Pichia***.

Second, and in further support the Appellants position, a Declaration by Dr. James Trager (referred to hereafter as "TD") was submitted during prosecution. A copy of the Trager Declaration is provided herewith as Appendix III. Under the case law and its own rules of practice, the Office is required to consider the factual evidence in the record, including the Trager Declaration and its factual underpinnings, and either accept them as true or rebut them with a factual showing of its own. *In re Alton*, 76 F.3d 1168, 1175, 37 U.S.P.Q.2d (BNA) 1578, 1583 (Fed. Cir. 1996).

In his career, Dr. Trager has worked with several different species of fungi, including *Saccharomyces cerevisiae* and *Pichia pastoris*, and has published several journal articles regarding this work. TD ¶3.

Further because Dr. Trager, in October 1999 (the filing date of this application), a) was one of skill in the art (termed by Dr. Trager as a "Skilled Person") and b) regularly attended external and internal meetings at which people of skill in the art presented their research, he is qualified by training and experience to address what one of skill in the would have understood from a reading of the cited publications, including Robinson. TD ¶7

Dr. Trager has reviewed all of the cited references and states that Robinson is the only reference that discusses dual-expression cassette vectors, and a suggestion to use such vector in "yeast" may be found in column 16 of Robinson. Dr. Trager states that a Skilled Person would not equate "yeast" with "*Pichia*" in Robinson, and, as such, a Skilled Person would find no suggestion to use dual expression cassette vectors for antibody production in *Pichia*. TD ¶10.

---

disclosed.").

Dr. Trager's reasoning for this position is set forth in TD ¶¶11-12.

Specifically, Dr. Trager states that as is known by the Skilled Person, the word "yeast" has one of two meanings, depending on the context of how it is used. In the first meaning, "yeast" solely refers to the species of *Saccharomyces cerevisiae*, commonly known as "brewer's yeast". For example, if a Skilled Person says he works in a "yeast lab", he is indicating that he works in a lab that works on *S. cerevisiae.* In the second meaning, "yeast" refers to a genus of fungi that encompasses over 25,000 species from the following families *Saccharomyes, Pichia, Candida, Schizosaccharomyces, Neurospora*, and others. As an example, throughout Dr. Trager's declaration the word "yeast" has been used in its second meaning, referring to a genus of fungi. In other words, depending on the context of how the word "yeast" is used in a reference, it refers to either *S. cerevisiae*, or a genus of over 25,000 species of fungi. TD ¶11.

In his declaration, Dr. Trager states that from the context in which the word "yeast" is used in Robinson, a Skilled Person would recognize that Robinson uses the word "yeast "with its first meaning -- as a reference to *S. cerevisiae.* A Skilled Person would recognize this because Robinson uses the terms, "yeast" and "*S. cerevisiae*" interchangeably. For example, Robinson refers to the *S. cerevisiae* gene as "the yeast invertase gene", refers to the *S. cerevisiae* PGK promoter as "the yeast PGK promoter", and refers to the origin of replication of the 2-micron plasmid endogenous to *S. cerevisiae* as "the yeast origin of replication, oriY, a cis-acting sequence (REP3) from the yeast endogenous 2-micron plasmid." According to Dr. Trager, at no point in the disclosure does Robinson suggest that "yeast" encompasses anything other than *S. cerevisiae.* ¶12

Dr. Trager concludes by stating that upon reading the Robinson reference as a whole, a Skilled Person would recognize that the "yeast" referred to by Robinson is, in fact, *S. cerevisiae,* and not a genus of fungi. Any suggestion by Robinson to use a dual expression cassette to express an antibody in yeast is, therefore, a suggestion to use a dual expression cassette vector to express an antibody in *S. cerevisiae.* Since *S. cerevisiae* and *Pichia* are different species, a Skilled Person would find no suggestion in Robinson to use dual expression cassette vectors for antibody production in *Pichia.* ¶13

Finally Dr. Trager finally states that based on his reasoning, it is his unequivocal opinion that a Skilled Person would find no suggestion in Robinson to use a dual expression cassette

vector for antibody production in *Pichia*. Further, Dr. Trager states that this suggestion is not provided by any of the other cited references. Accordingly, the cited references, independently or together, do not suggest using a dual expression cassette vector for use in *Pichia*. ¶14

In view of the foregoing arguments, the Appellants respectfully submit that the Office, at best, has provided a suggestion to use a dual expression cassette vector to express an antibody in *S. cerevisiae*, not *Pichia*. Since the claims are directed to methods and compositions for producing antibodies using a dual expression cassette vector in *Pichia*, not *S. cerevisiae*, the Appellants respectfully submit that the Office has failed to establish a *prima facie* case of obviousness. Accordingly, this rejection should be withdrawn.

b) The art provides no reasonable expectation of success

Even if Robinson were to suggest use of dual expression cassette vectors in *Pichia* – and it is Appellants' unequivocal position that Robinson does not provide any such suggestion -- the Appellants respectfully submit that the cited art further provides no reasonable expectation of success in the practice of the claimed invention.

In support of this position, the Appellants respectfully submit that scientific literature relating to antibody expression in *Pichia* would actually lead a Skilled Person away from combining the cited references. For example, two reviews of the scientific literature on antibody expression in *Pichia* each discourage the Skilled Person from the expression of antibodies in *Pichia* using dual expression cassette vectors. These references are submitted herewith as part of an Information Disclosure Statement, and are part of the record of this application. For the Board's convenience, copies of these references are again submitted with this Brief as Appendices III and IV, and the sections of the references referred to below are underlined.

The first of these references, Pennell (*Res Immunol.* 1998 149:599-603; Appendix III), states "The size of the protein to be expressed may also be limiting because to our knowledge, there are no reports of proteins greater than 117 kDa being expressed in *P. pastoris*." (emphasis added). Since antibodies are generally larger than 117 kDa, Pennell's disclosure would lead a Skilled Person away from expressing a whole antibody in *Pichia*.

The second of these references, Holliger (*Methods in Mol Biol.* 2002 178:348-357; Appendix IV), states, in section 8 on page 351 "Because bicistronic expression works only poorly in *Pichia* (unlike *E. coli*), it is preferable to use single-chain Ab formats. Two chain Ab formats require that the two chains be cloned and transformed separately". (emphasis added). Hollinger, therefore, unequivocally and explicitly states that *single* expression cassette vectors are **required** if expression of two different chains of an antibody is desired.

As such, in view of these references, especially in view of Hollinger's explicit warning against dual expression vectors for antibody in *Pichia*, a skilled person would be directly led away from combining the cited references to provide the invention.

Dr. Trager has reviewed these references, and has expressed agreement with the Appellants' position in his declaration. TD ¶¶17-21. Dr. Trager summarizes his analysis by stating: "it is my unequivocal opinion that a Skilled Person, in view of the cited publications (i.e., Robinson et al, *etc.*), would not find the invention obvious because the literature and common knowledge in the field would lead them away from doing so. Given that two different reviews of the field of antibody expression in *Pichia* categorically and in no uncertainty direct away from using dual expression cassette vectors, why would a Skilled Person expect it would work?" TD ¶22

Furthermore, the Appellants respectfully submit that protein expression is unpredictable, and successful heterologous protein expression in *S. cerevisiae* does not predict successful heterologous protein expression in *Pichia*.

Support for this assertion is found in Dr. Trager's Declaration.

Dr. Trager states in his declaration that *S. cerevisiae* and *Pichia* are very different, phylogenetically distinct, species. It follows that a Skilled Person would recognize that even if a protein could be expressed in one species, there would be no reasonable expectation of success that it could be expressed in the other. For example, even if functional antibodies were shown to be expressed in *S. cerevisiae*, a Skilled Person would have no reasonable expectation of success in expressing the same antibodies in *Pichia*. TD ¶15

Dr. Trager concludes by stating that a Skilled Person would recognize that an example of expressing a heterologous protein, such as an antibody, in *S. cerevisiae* would have no bearing

whatsoever on whether or not that same heterologous protein could be expressed in *Pichia*. Even if a reference was cited that actually showed a working method for the expression of functional antibodies in *S. cerevisiae* using a dual expression cassette vector, it is my unequivocal opinion that a Skilled Person would have no reasonable expectation of success in practicing such a method in *Pichia*. TD ¶16

Robinson fails to show expression of an antibody in *S. cerevisiae* using a dual expression cassette vector. Thus, the ordinarily skilled artisan must make at least three leaps from the disclosure of Robinson – 1) that "yeast" means something other than *S. cerevisiae* (as discussed above); 2) that evidence of a single expression cassette vector for antibody production works in *S. cerevisiae* is predictive of success in using a ***dual*** expression cassette vector for antibody production in *S. cerevisiae*; and 3) expression in *S. cerevisiae* can be reasonably extrapolated to predict success in expression in *Pichia*. The reviews provided in Appendices III and IV, combined with Dr. Trager's declaration, provides ample evidence that none of these leaps are trivial, and that the ordinarily skilled artisan would not make these leaps.

Accordingly, even if one of skill in the art *were* to interpret the word "yeast" broadly to encompass the species *Pichia*, and even if Robinson did teach successful expression of an antibody in *S. cerevisiae* using a dual expression cassette, one of skill in the art would still not be motivated to use a dual expression cassette vector for antibody expression in *Pichia,* since, as noted by Dr. Trager, there would have been no reasonable expectation of success in practicing such a method in *Pichia.*

In summary, one of skill in the art would find no specific motivation to combine the cited references to provide the claimed invention, and, in fact would be strongly led away from the invention.

The Appellants respectfully submit that the foregoing discussion adequately addresses this rejection of the appealed claims. Withdrawal of the rejection.is respectfully requested.

c Specific assertions by the Examiner

In the Office Action of September 12, 2003, the Examiner remained unpersuaded by the Appellants' arguments.

In response to the Appellants arguments that the word "yeast" means "S. cerevisiae" in Robinson, the Examiner stated that the arguments were unpersuasive because "the term "yeast" can encompass Pichia and in fact does". Office Action ¶4 on page 3. The Appellants respectfully submit that the Examiner's statement is not accurate because, as argued above, the term "yeast" has two meanings, and depending on its meaning, may *not* encompass *Pichia*. In fact, as used in Robinson, the word "yeast" does *not* encompass *Pichia*.

Further, the Examiner finds the Appellants' arguments unconvincing because "one reading the art of the Invitrogen catalog in combination with the cited references would have the motivation to use the Pichia strain as well as the expression vectors described therein because of the *benefits recited for the Pichia expression system catalog*" ¶4 on page 3. (Emphasis added). However, as reasoned above, there is no suggestion in any of the references, including the "Pichia expression system catalog" (which is interpreted to mean the cited Invitrogen catalog), to use a dual expression cassette vector in *Pichia*. To be explicitly clear: the Invitrogen catalog does not even disclose a dual expression cassette vector. How can the Invitrogen catalog provide any motivation to use a dual expression cassette vector to produce antibodies in *Pichia* if such a vector is not even disclosed?

Further, the Examiner finds the Appellants' arguments regarding the teachings of Holliger unconvincing because "the citation is in a reference and it is unclear what is meant by the citation", "there is no scientific evidence provided in the response to warrant the need to use two separate vectors", and because Dr. Trager uses the phrase "Holliger, therefore *appears* to say that.....". (emphasis added) Office Action ¶4 on pages 4 and 5.

In rebuttal, the Appellants respectfully submit that Holliger (*Methods in Mol Biol.* 2002 178:348-357) represents a detailed review of methods for expressing antibody fragments in *Pichia pastoris*. It is one of very few reviews on antibody expression in *Pichia*, and a reference that one of skill in the art would undoubtedly turn to when deciding an approach for producing an antibody in *Pichia*.

In Holliger's materials section, Holliger provides a list of reagents for use in antibody production in *Pichia*. The antibody vector "Ab clone" is detailed in section 8 on page 351. In this section, Holliger states "Two-chain Ab formats (e.g., Fvs, Fabs, bispecific diabodies) require that the two chains be cloned and transformed separately". This statement is clear and concise, and, even in the absence of data to support Holliger's advice, Holliger's advice represents a significant teaching away from the claimed invention, which requires that the two chains be cloned on the same vector, not separately.

The Examiner also asserts that Dr. Trager, because he uses the phrase "Holliger, therefore *appears* to say that..." (emphasis added), is indicating ambiguity as to the meaning of Holliger's advice. However, Dr. Trager also states that "two different reviews of the field of antibody expression in *Pichia* [one being Holliger and the other Pennell] categorically and in no uncertainty direct away from using dual expression cassette vectors". (phrase in brackets added) SD ¶ 22. The Appellants respectfully submit that any ambiguity in Dr. Trager's opinion of the teachings of Holliger are addressed by this later unqualified statement.

Accordingly, the Examiner's reasoning for dismissing the Appellants' submissions, including their arguments and the Trager Declaration, lack force.

II. THE INVENTION AS CLAIMED IN CLAIMS 36-39 AND 41-50 IS NOT OBVIOUS UNDER 35 U.S.C. §103 IN VIEW OF HORWITZ, CREGG, THE INVITROGEN CATALOG, ROBINSON, AND VANDERLAAN

Claims 36-39 and 41-50 stand rejected as *prima facie* obvious in view of Horwitz, Cregg, The Invitrogen Catalog and Robinson (Issue I) and further in view of Vanderlaan.

As noted above, the two issues on appeal are each rejections of the claims under 35 U.S.C. §103(a) which are based on a similar combination of references. The rejection of Issue I is based on a combination of the disclosures of Horwitz, Cregg, The Invitrogen Catalog and Robinson. Issue II addressed here is based on a combination of those same references further combined with Vanderlaan.

The errors in the rejection here are the same as those set out above for Issue I. Vanderlaan does nothing to cure the deficiencies of the rejection. Rather Vanderlaan only discloses an antibody that binds dioxin, and thus is asserted to provide the element of rejected claim 41.

However, Vanderlaan is silent with respect to expression of an antibody in *Pichia* or the use of a dual expression cassette vector.

The Appellants respectfully submit that the discussion above adequately addresses this rejection of the appealed claims. Withdrawal of the rejection is respectfully requested.

SUMMARY

Contrary to the Examiner's assertions, the Appellants respectfully submit that the claims are not obvious in view of the cited art because: a) the desirability of ***dual*** expression cassette vectors for antibody production in ***Pichia*** is not suggested by the cited art, and b) one of skill in the art would not practice a method of producing antibodies in *Pichia* using a dual expression cassette vector with any reasonable expectation of success because the art directly teaches directly away from using dual-expression cassette vectors, and because it would not be possible to predict is such a method would work in *Pichia* prior to performing the method.

RELIEF REQUESTED

Appellants respectfully request that the rejection of claims 36-39 and 41-50 under 35 U.S.C. §103 be reversed, and that the application be remanded to the Examiner with instructions to issue a Notice of Allowance.

REQUEST FOR ORAL HEARING

Appellants request an oral hearing on this appeal, and enclose two additional copies of this Brief in connection therewith.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: February 23, 2004

By: James S Keddie
James S. Keddie
Registration No. 48,920

BOZICEVIC, FIELD & FRANCIS LLP
200 Middlefield Road, Suite 200
Menlo Park, California 94025
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

F:\DOCUMENT\UCAL\269\!!appeal brief.doc

APPENDIX I

PENDING CLAIMS

36. A method for production of an antibody that specifically binds an antigen of interest, the method comprising the steps of:

culturing a recombinant *Pichia* cell, the cell comprising a vector comprising a first and a second expression cassette, wherein:

said first expression cassette comprises a first promoter operably linked to a nucleic acid encoding an immunoglobulin light chain operably linked to a first signal peptide;

said second expression cassette comprises a second promoter operably linked to a nucleic acid encoding an immunoglobulin heavy chain operably linked to a second signal peptide,

and said culturing provides for expression of the immunoglobulin light and heavy chains; and

harvesting specific antigen-binding antibody from culture supernatant, which antibody specifically binds an antigen of interest.

37. The method of Claim 36, wherein each of said first and second signal sequences is a yeast α-factor signal sequence.

38. The method of Claim 37, wherein each of said first and second signal sequences is a *Saccharomyces cerevisiae* α-factor signal sequence.

39. The method of Claim 36, wherein said *Pichia* is *Pichia pastoris*.

41. The method of Claim 36, wherein said antibody specifically binds dioxin.

42. The method of Claim 36, wherein each of said first and second promoters is an inducible promoter.

43. The method of Claim 42, wherein each of said first and second promoters is an alcohol oxidase promoter.

44. The method of Claim 43, wherein said each of first and second promoters is a *Pichia* alcohol oxidase promoter.

45. The method of Claim 36, wherein the antibody is a mouse antibody, a humanized mouse antibody, or a human antibody.

46. The method of Claim 36, wherein the antibody is recovered from the culture supernatant at more than about 10mg/l.

47. A *Pichia* expression vector comprising:

a first and a second expression cassette, said first cassette comprising a first promoter operably linked to a nucleic acid encoding an immunoglobulin light chain operably linked to a first signal peptide, and said second cassette comprising a second promoter operably linked to a nucleic acid encoding an immunoglobulin heavy chain operably linked to a second signal peptide,

wherein introduction of said vector into a *Pichia* host cell provides for production of a recombinant immunoglobulin protein that specifically binds an antigen and is secreted by the host cell.

48. A recombinant *Pichia* cell containing the expression vector of claim 47.

49. A method for production of an antibody comprising the steps of:

culturing the recombinant *Pichia* cell of claim 48 so as to provide for antibody expression; and

harvesting the antibody from culture supernatant.

50. A method for production of an antibody that specifically binds an antigen of interest, the method comprising the steps of:

culturing a recombinant *Pichia* cell, the cell comprising a vector comprising a first and a second expression cassette, wherein:

said first expression cassette comprising a *Pichia* alcohol oxidase promoter operably linked to a nucleic acid encoding an immunoglobulin light chain operably linked to a first signal peptide;

said second expression cassette comprising a *Pichia* alcohol oxidase promoter operably linked to a nucleic acid encoding an immunoglobulin heavy chain operably linked to a second signal peptide, and said culturing provides for expression of the immunoglobulin light and heavy chains; and

harvesting specific antigen-binding antibody from culture supernatant, which antibody specifically binds an antigen of interest.

Appendix II

DECLARATION OF JAMES B TRAGER UNDER 37 C.F.R. § 1.132 copy for Appeal Brief	Attorney Docket	UCAL-269
	First Named Inventor	P. Choudary
	Confirmation Number	9044
	Application Number	09/425,075
	Filing Date	October 21, 1999
	Group Art Unit	1642
	Examiner Name	Larry Helms
	Title: *FUNCTIONALLY ASSEMBLED ANTIGEN-SPECIFIC INTACT RECOMBINANT ANTIBODY AND A METHOD FOR PRODUCTION THEREOF*	

Dear Sir:

1. I, James B. Trager, declare and say I am a resident of the State of California. My residence address is 1308 Park Avenue, Alameda, California.

2. I hold a B.A. degree in Philosophy, which I received from St. John's College, Santa Fe, in 1984. I further hold an Ph.D. degree, which I received from the University of California at Berkeley, in 1994. I currently work as a Senior Scientist at Geron Corporation.

3. I did my Ph.D. in the field of gene expression in *Saccharomyces cerevisiae*, and, during the course of my research career, I have worked with several different species of yeast, including *Saccharomyces cerevisiae* and *Pichia pastoris*, sometimes for expression of heterologous proteins. Details of my career and publications may be found in my *curriculum vitae*, provided herewith. I am therefore very familiar with yeast in general, and am well qualified to offer my opinion on what a researcher of ordinary skill in the art would consider obvious in the area of antibody expression in *Pichia* in October 1999, the filing date of this application.

4. I have reviewed the claims, and understand that the claimed invention (i.e., the "Invention" is a method for expressing antibodies in *Pichia* using a dual expression cassette vector, and vectors and host cells for performing the method. I have also reviewed the publications (i.e., the "cited publications") of Robinson et al., Horwitz *et al*, Cregg *et al*, The Invitrogen Catalog 1997, and Sambrook *et al,* as cited in the rejection set forth in the Office Action dated April 16, 2003.

5. I have been asked to opine of the following general question:

In view of the cited publications, would one of skill in the art, in October 1999, think that a method for expressing antibodies in *Pichia* using a dual expression cassette vector is obvious?

It is my unequivocal opinion, based on the facts and reasoning set forth below, that the answer to this question is "no".

6. It is my understanding that the cited publications are to be viewed from the standpoint of one of ordinary skill in the art in the relevant field (a "Skilled Person") at the time of filing of the patent application in question. The patent application in question was filed on October 21, 1999, and relates to the field of heterologous protein expression in yeast. I would expect a Skilled Person in the field of heterologous protein expression in yeast, immediately prior to and up to October, 1999 (the "relevant period") to have been represented by a scientist with a Ph.D. degree. I consider that such a Skilled Person would have the ability to make constructs for expressing heterologous proteins in yeast without inventive effort.

7. Since during the relevant period I a) was a Skilled Person and b) regularly attended external and internal meetings at which Skilled Persons presented their

research, I believe that I am qualified by training and experience to address what a Skilled Person would have understood from a reading of the cited publications.

8. There are three main bases for my opinion that a Skilled Person would not conclude that the Invention is obvious in view of the cited publications. I will detail those reasons below:

The cited references, independently or together, do not suggest using a dual expression cassette vector for use in *Pichia*

9. The cited references, independently or together, do not suggest using a dual expression cassette vector for use in *Pichia*. The reasoning behind this statement is set forth below:

10. Robinson is the only reference that discusses dual-expression cassette vectors, and a suggestion to use such vector in "yeast" may be found in column 16 of Robinson. A Skilled Person would not equate "yeast" with "*Pichia*" in Robinson, and, as such, a Skilled Person would find no suggestion to use dual expression cassette vectors for antibody production in *Pichia*.

11. As is known by the Skilled Person, the word "yeast" has one of two meanings, depending on the context of how it is used. In the first meaning, "yeast" solely refers to the species of *Saccharomyces cerevisiae*, commonly known as "brewer's yeast". For example, if a Skilled Person says he works in a "yeast lab", he is indicating that he works in a lab that works on *S. cerevisiae*. In the second meaning, "yeast" refers to a genus of fungi that encompasses over 25,000 species from the following families *Saccharomyes, Pichia, Candida, Schizosaccharomyces, Neurospora*, and others. As an example, throughout this declaration I have used the word "yeast" in its second meaning, referring to a genus of fungi. In other words, depending on the context of

how the word "yeast" is used in a reference, it refers to either *S. cerevisiae*, or a genus of over 25,000 species of fungi.

12. From the context of how the word "yeast" is used in Robinson, a Skilled Person would recognize that Robinson uses the word yeast with its first meaning, as a reference to *S. cerevisiae*. A Skilled Person would recognize this because Robinson uses the terms, "yeast" and "*S. cerevisiae*" interchangeably. For example, Robinson refers to the *S. cerevisiae* gene as "the yeast invertase gene", refers to the *S. cerevisiae* PGK promoter as "the yeast PGK promoter", and refers to the origin of replication of the 2-micron plasmid endogenous to *S. cerevisiae* as "the yeast origin of replication, oriY, a cis-acting sequence (REP3) from the yeast endogenous 2-micron plasmid." At no point in the disclosure does Robinson suggest that "yeast" encompasses anything other than *S. cerevisiae*.

13. Upon reading the Robinson reference as a whole, a Skilled Person would recognize that the "yeast" referred to by Robinson is, in fact, *S. cerevisiae,* not a genus of fungi. Any suggestion by Robinson to use a dual expression cassette to express an antibody in yeast, is, therefore, a suggestion to use a dual expression cassette to express an antibody in *S. cerevisiae*. Since *S. cerevisiae* and *Pichia* are different species, a Skilled Person would find no suggestion in Robinson to use dual expression cassette vectors for antibody production in *Pichia*.

14. Based on the reasoning set forth above, it is my unequivocal opinion that a Skilled Person would find no suggestion in Robinson to use a dual expression cassette vector for antibody production in *Pichia*. Since this suggestion is not provided by any of the other cited references, the cited references, independently or together, do not suggest using a dual expression cassette vector for use in *Pichia*.

Successful heterologous protein expression in *S. cerevisiae* does not predict successful heterologous protein expression in *Pichia*

15. *S. cerevisiae* and *Pichia* are very different, phylogenetically distinct, species. It follows that a Skilled Person would recognize that even if a protein could be expressed in one species, there would be no reasonable expectation of success that it could be expressed in the other. For example, even if functional antibodies were shown to be expressed in *S. cerevisiae*, a Skilled Person would have no reasonable expectation of success in expressing the same antibodies in *Pichia*.

16. As such, a Skilled Person would recognize that an example of expressing a heterologous protein, such as an antibody, in *S. cerevisiae* would have no bearing whatsoever on whether or not that same heterologous protein could be expressed in *Pichia*. Even if a reference was cited that actually showed a working method for the expression of functional antibodies in *S. cerevisiae* using a dual expression cassette vector, it is my unequivocal opinion that a Skilled Person would have no reasonable expectation of success in practicing such a method in *Pichia*.

The art would lead a Skilled Person from combining the cited references

17. The Invention involves a dual expression cassette vector for expression of immunoglobulin heavy and light chains of an antibody. Because two expression cassettes are on the same vector, a Skilled Person, would not have any reasonable expectation of success in making and using such a vector because of the problems associated with intra-molecular recombination (*e.g.* occurring when two parts of a vector are similar in nucleotide sequence), transcriptional interference (*e.g.* occurring when transcription of one expression cassette does not terminate properly, so that transcription "reads through" so as to interfere with transcription of the second expression cassette), and translational interference (*e.g.* occurring when

transcriptional read-through of the first expression cassette produces an antisense molecule that interferes with the translation of the RNA from the second expression cassette). Such problems are commonly associated with such dual expression cassette vectors, especially when the expression cassettes contain polynucleotides with similar or identical sequences (for example similar promoters, signal sequence-encoding polynucleotides or terminators). The usual way of making vectors usually involves an intermediate vector production step in bacteria (*e.g.*, *E. coli*) these problems would pose serious technical barriers. Thus, even if these problems only happened in bacteria, they would still impact the question of whether or not a Skilled Person would make and use such a vector.

18. The following publications support this position: Hoshizaki (*Mol Cell Bio*, 1985 5:3323-9), Peterson (*J. Bact.*, 1983 156: 177-85); Nies (*J. Antimicrob Chemother.* 1986 18:Suppl 35-41); and a page of technical material found at Stratagene's website (http://www.stratagene.com/displayProduct.asp?productId=290). I am told that these publications have been previously provided to the Examiner.

19. Further, with specific reference to antibody expression in *Pichia*, there appears to be a significant amount of scientific literature that would lead a Skilled Person away from combining the cited references. For example, two reviews of the scientific literature on antibody expression in *Pichia* each discourage the Skilled Person from the expression of antibodies in *Pichia* using dual expression cassette vectors.

20. The first of these references, Pennell (*Res Immunol.* 1998 149:599-603; Exhibit A), states "The size of the protein to be expressed may also be limiting because to our knowledge, there are no reports of proteins greater than 117 kDa being expressed in *P. pastoris*." Since antibodies are generally larger than 117 kDa, Pennell's disclosure would lead a Skilled Person away from expressing a whole antibody in *Pichia*.

21. The second of these references, Holliger (*Methods in Mol Biol.* 2002 178:348-357; Exhibit B), states, in point 8 on page 351 "Because bicistronic expression works only poorly in *Pichia* (unlike *E. coli*), it is preferable to use single-chain Ab formats. Two chain Ab formats require that the two chains be cloned and transformed separately". (Underlining added). Hollinger, therefore, appears to say that single expression cassette vectors are **required** if expression of two different chains of an antibody is desired.

22. Based on the foregoing discussion, it is my unequivocal opinion that a Skilled Person, in view of the cited publications (i.e., Robinson et al, *etc.*), would not find the invention obvious because the literature and common knowledge in the field would lead them away from doing so. Given that two different reviews of the field of antibody expression in *Pichia* categorically and in no uncertainty direct away from using dual expression cassette vectors, why would a Skilled Person expect it would work?

23. In summary, and in view of the technical problems and guidance set out in the scientific literature as exemplified by the reviews discussed above, it is my unequivocal opinion that a Skilled Person would not conclude that the Invention is obvious in view of the cited publications. A Skilled Person would find no specific motivation to combine the cited publications to provide the Invention, and, in fact would be strongly led away from the invention.

24. I acknowledge I have been paid $250 for my services in reviewing the materials described herein, and in rendering this opinion.

25. I, James B. Trager, hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge

that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

July 9, 2003
Date

James B. Trager, Ph.D.

Attachments:

Exhibit A: Holliger, *Methods in Mol Biol.* 2002 178:348-357
Exhibit B: Pennell, *Res Immunol.* 1998 149:599-603

F:\DOCUMENT\UCAL\269\Declaration Trager 1.132.doc

Ward, E.S., Gussow, D., Griffiths, A.D., Jones, P.T. & Winter, G. (1989), Binding activities of a repertoir of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature*, 341, 544-546.

Ward, M., Wilson, L.J., Kodama, K.H., Rey, M.W. & Berka, R.M. (1990), Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. *Bio/Technology*, 8, 435-440.

Wu, X.C., Ng, S.C., Near, R.I. & Wong, S.L. (1993), Efficient production of a functional single-chain antidigoxin antibody via an engineered *Bacillus subtilis* expression-secretion system. *Bio/Technnology*, 11, 71-76.

Yu, H., Nakano, Y., Yamashita, Y., Oho, T. & Koga, T. (1997), Effects of antibodies against cell surface protein antigen PAc-glucosyltransferase fusion proteins on glucan synthesis and cell adhesion of *Streptococcus mutans*. *Infect. Immun.*, 65, 2292-2298.

In vitro production of recombinant antibody fragments in *Pichia pastoris*

C.A. Pennell [1] [*] and P. Eldin [2]

[1] *Department of Laboratory Medicine and Pathology & University of Minnesota Cancer Center, University of Minnesota, Minneapolis, MN 55455 (USA), and*
[2] *Dynamique Moleculaire des Interactions membranaires, Montpellier, Cedex 5 (France)*

Introduction

The methyltropic yeast *Pichia pastoris* is rapidly becoming a preferred host for the efficient expression of heterologous proteins (reviewed in Hollenberg and Gellissen, 1997; Sreekrishna *et al.*, 1997; Sudbery, 1996). *P. pastoris* combines the general features of protein expression in eukaryotes with the fast growth and genetic modifiability of prokaryotes. It is less expensive than other eukaryotic expression systems, such as baculovirus or mammalian tissue culture, and it typically yields higher quantities of secreted functional proteins. The ability to correctly express folded secreted proteins, including highly disulphide-bonded ones (White *et al.*, 1994), provides a distinct advantage over bacterial systems that often require laborious and inefficient procedures to denature and refold proteins expressed as insoluble, inclusion bodies (Skerra, 1993). *P. pastoris* is a superior expression host over its more famous non-methyltropic relative, *Saccharomyces cerevisiae*, because *P. pastoris* grows stably to high cell densities in fermentors (> 100 mg dry weight per ml culture) and it has strong, tightly regulated promoters (Ellis *et al.*, 1985). For these reasons, *P. pastoris* has recently been exploited as an expression system for the high-level secretion of many proteins, including recombinant antibody fragments.

By definition, methyltropic yeasts are capable of utilizing methanol as their sole carbon source. The first enzyme in the methanol-utilization pathway, alcohol oxidase (AOX), is encoded by two closely related genes: *AOX1* and *AOX2* (Ellis *et al.*, 1985; Cregg *et al.*, 1989). Although the AOX1 and AOX2 proteins have 97% sequence identity and equivalent enzymatic activity, over 95% of the alcohol oxidase activity in *P. pastoris* is attributable to AOX1. This is due to the strength of the *AOX1* promoter (Cregg *et al.*, 1989; Koutz *et al.*, 1989). Upon the addition of methanol, *AOX1* gene transcription is rapidly induced to high levels and ultimately accounts for 5% of the total polyA$^+$ RNA (Cregg and Madden, 1988). The AOX1 protein is correspondingly overexpressed and comprises up to 30% of the total intracellular protein. Heterologous genes are therefore cloned under control of the *AOX1* promoter to

Received July 10, 1998.

(*) To whom correspondence should be addressed.

allow for their rapid and strong induction by methanol. The expression of heterologous genes driven by the *AOX1* promoter can result in protein yields exceeding 200 mg/l in shake flask cultures, and can be in the g/l range in fermentation cultures (Faber *et al.*, 1995; Cregg *et al.*, 1993).

Generating and screening transformants

To maximize the stability of protein expression, heterologous genes are integrated into the *P. pastoris* genome. A series of expression vectors designed for homologous integration are commercially available (Invitrogen, San Diego, USA; www.invitrogen.com). The salient features of these vectors are the inclusion of the 5' and 3' regions of the *AOX1* gene (to target plasmid integration via homologous recombination), transcription termination and polyadenylation signals, and the *HIS4* gene (for selecting yeast transformants able to grow in histidine-deficient media) or the Zeocin resistance gene (for selecting both bacterial and yeast transformants). Some vectors contain additional features such as the f1 origin of replication (for mutagenesis of single-stranded DNA), the kanamycin resistance gene (for selecting G418-resistant high copy number transformants), or yeast-derived signal sequences (for protein secretion). Plasmids containing the gene of interest are linearized and then integrated in the yeast genome via homologous recombination upon transformation by spheroplasting or electroporation. We routinely use electroporation because of its simplicity and relatively high efficiency (10^3-10^4 transformants per μg of DNA).

The usual goal of screening transformants is to identify high protein-expressing "jackpot" clones. Although expression levels are affected by the integration site, gene sequence, and the strain of *P. pastoris* transformed, the most profound effect typically results from copy number (Clare *et al.*, 1991). High copy number integrants are identified genetically by PCR (Linder *et al.*, 1996; Haaning *et al.*, 1997) or immunologically with specific antibodies (McGrew *et al.*, 1997; Wung and Gascoigne, 1996). Screening procedures such as these can be combined with genetic or selection strategies designed to enrich for high copy integrants. For example, plasmids with multicopy inserts can be generated prior to transformation, or high copy number integrants can be selected for their increased resistance to the drug G418 if the integrated plasmid contains the kanamycin resistance gene (Scorer *et al.*, 1994).

Secreted vs. intracellular expression

Induced proteins are expressed intracellularly or are secreted, depending on the absence or presence of an appropriate signal sequence. The utility of exploiting the secretory pathway is that *P. pastoris* can be grown to high cell densities, and then induced with methanol, in inexpensive, chemically defined, protein-poor media. The low level of protein in the media formulations facilitates detection and purification of the final secreted product, which can comprise the vast majority (80-90%) of the total protein in the supernatant (Faber *et al.*, 1995). Secreted proteins are easily and efficiently purified from the supernatants, often in one step by affinity or metal-chelating chromatography (Eldin *et al.*, 1997).

Expression of recombinant antibody fragments

The first recombinant antibody fragment reported to be expressed in *P. pastoris* was a rabbit single chain Fv (scFv) selected from a bacterial phage display library (Ridder *et al.*, 1995). ScFv fragments contain heavy and light chain variable regions connected by a small, flexible peptide (Huston *et al.*, 1988; Bird *et al.*, 1988). These fragments can be modified to increase their stability or avidity (e.g. disulphide-stabilized scFv and bivalent scFv fragments), and to add additional specificities or effector functions (e.g. bispecific diabodies or scFv-immunotoxins) (table I). ScFv fragments are well suited for many *in vivo* diagnostic and therapeutic applications because their reduced size (27,000-30,000 M_r) permits them to penetrate tissues more rapidly than whole antibodies and to be cleared more rapidly from the blood (Yokota *et al.*, 1992). Because the *in vivo* use of scFv fragments and their derivatives often requires large quantities of protein, it is not surprising that most antibody fragments expressed in *P. pastoris* have clinical potential.

The reported levels of scFv production in *P. pastoris* range from 10 to 250 mg/l in shake-flask cultures (table I). These fragments are almost invariably expressed as secreted proteins in minimal media to facilitate their purification. However, Luo *et al.* (1997b) recently reported that a scFv fragment was induced to comparable levels (200 mg/l) in yeast grown in the standard buffered minimal methanol medium or in a completely protein-free medium. The ability to produce large amounts of secreted proteins in essentially phosphate buffer should make large-scale protein preparations in *P. pastoris* even more attractive.

Disadvantages of protein expression in *P. pastoris*

Not every protein can be expressed to high levels in *P. pastoris* due to factors such as codon bias and the requirement for particular post-translational modifications (Sreekrishna *et al.*, 1997). However, shuttle vectors exist that allow the same gene to be expressed in bacterial, yeast, or mammalian cells

Table I. Antibody fragments secreted by *P. pastoris.*

Ab fragment	Antigen specificity	Yield	Reference
scFv	Leukaemia inhibitory factor	> 100 mg/l	(Ridder *et al.*, 1995)
scFv	Squamous carcinoma	10-50 mg/l	(Luo *et al.*, 1995; Luo *et al.*, 1996)
dsFv		NR(*)	
scFv-chelator		NR	
dsFv-chelator		NR	
scFv	Thomsen-Friedenreich (pan-adenocarcinoma)	200 mg/l	(Luo *et al.*, 1997b)
bivalent scFv	CA125-ovarian carcinoma	100 mg/l	(Luo *et al.*, 1997a)
bivalent diabody	Carcinoembryonic antigen	1 mg/l	(FitzGerald *et al.*, 1997)
bispecific diabody	CEA/CD3	1 mg/l	
scFv	Desipramine	250 mg/l	(Eldin *et al.*, 1997)
scFv	CD7	60 mg/l	
scFv/B7-2	erbB2/CD28	0.5 mg/l	(Gerstmayer *et al.*, 1997)

(*) Not reported.

(White *et al.*, 1994, 1995; Liu *et al.*, 1998). These vectors permit investigators to choose the expression system that best fits their particular need, be it high-yield or appropriate post-translation modification, with minimal genetic manipulations. The size of the protein to be expressed may also be limiting because to our knowledge, there are no reports of proteins greater than 117 kDa (β-galactosidase) being expressed in *P. pastoris.* High-yield protein production in *P. pastoris* is dependent on several factors such as adequate aeration, methanol concentration and temperature maintenance at 28°-30°C (*P. pastoris* is not thermotolerant). All of these conditions have to be maintained to ensure optimal inductions but recent advances in batch-fed fermentation (White *et al.*, 1995; Chiruvolu *et al.*, 1997; Jimenez *et al.*, 1997; Chen *et al.*, 1997) and shake-flask technologies (Guarna *et al.*, 1997) should facilitate monitoring induction conditions.

Conclusions

We have found *P. pastoris* to be an inexpensive and robust system for the high-level production of antibody fragments. To find that "jackpot" clone, we recommend the following strategy: clone the relevant gene into a plasmid that contains a signal sequence, the kanamycin resistance gene, and an epitope- or histidine-tag sequence, select for transformants with increased G418 resistance, and then screen colony lifts of induced transformants with tag-specific antibodies. An alternate strategy is to linearize the plasmid separately with different restriction endonucleases to target integration at various sites in the *Pichia* genome. Differentially linearized vectors could be mixed in the same transformation to generate multiple integrants per clone. This increases the likelihood that the gene of interest integrates in a transcriptionally active site dependent on local chromatin structure.

A recent report of alpha-sarcin ribotoxin production in *P. pastoris* suggests that this system may soon be exploited for the expression of scFv immunotoxins (Martinezruiz *et al.*, 1998). Larger, intact antibodies such as camelid heavy chain IgG (Hamers-Casterman *et al.*, 1993) might also be expressed, since *P. pastoris* can secrete functional disulphide-bonded homodimers and heterodimers (Luo *et al.*, 1997a; FitzGerald *et al.*, 1997; Kalandadze *et al.*, 1996). We therefore anticipate that the use of *P. pastoris* as an expression system for antibody-derived fragments will be on the rise.

Acknowledgments

This work was sponsored in part by a postdoctoral fellowship from Elf Aquitaine, Inc. to P.E. and by grants to C.A.P. from the National Cancer Institute (CA-59510) and the Leukemia Task Force.

References

Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.-M., Lee, T., Pope, H.S., Riordan, G.S. & Whitlow, M. (1988), Single-chain antigen-binding proteins. *Science*, 242, 1038-1041.

Chen, Y.L., Cino, J., Hart, G., Freedman, D., White, C. & Komives, E.A. (1997), High protein expression in fermentation of recombinant *Pichia pastoris* by a fed-batch process. *Process Biochem.*, 32, 107-111.

Chiruvolu, V., Cregg, J.M. & Meagher, M.M. (1997), Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fedbatch fermentations. *Enzyme Microb. Technol.*, 21, 277-283.

Clare, J.J., Rayment, R.B., Ballantine, S.P., Sreekrishna, K. & Romanos, M.A. (1991), High level expression of tetanus toxoid fragment C in *Pichia pastoris* strains containing multiple tandem integrants of the gene. *Bio/Tech.*, 9, 455-460.

Cregg, J.M. & Madden, K.R. (1988), Development of the methyltropic yeast, *Pichia pastoris*, as a host system for the production of foreign proteins. *Dev. Ind. Microbiol.*, 29, 33-41.

Cregg, J.M., Madden, K.R., Barringer, K.J., Thill, G.P. & Stillman, C.A. (1989), Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*. *Mol. Cell. Biol.*, 9, 1316-1323.

Cregg, J.M., Vedvick, T.S. & Raschke, W.C. (1993), Recent advances in the expression of foreign genes in *Pichia pastoris*. *Bio/Tech.*, 11, 905-910.

Eldin, P., Pauza, M.E., Hieda, Y., Lin, G.F., Murtaugh, M.P., Pentel, P.R. & Pennell, C.A., (1997), High-level secretion of two antibody single chain Fv fragments by *Pichia pastoris*. *J. Immunol. Methods*, 201, 67-75.

Ellis, S.B., Brust, P.F., Koutz, P.J., Waters, A.F., Harpold, M.M. & Gingeras, T.R. (1985), Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast, *Pichia pastoris*. *Mol. Cell. Biol.*, 5, 1111-1121.

Faber, K.N., Harder, W., Ab, G. & Veenhuis, M. (1995), Methylotrophic yeasts as factories for the production of foreign proteins. *Yeast*, 11, 1331-1344.

FitzGerald, K., Holliger, P. & Winter, G. (1997), Improved tumour targeting by disulphide stabilized diabodies expressed in *Pichia pastoris*. *Protein Eng.*, 10, 1221-1225.

Gerstmayer, B., Altenschmidt, U., Hoffmann, M. & Wels, W. (1997), Costimulation of T cell proliferation by a chimeric B7-2 antibody fusion protein specifically targeted to cells expressing the erbB2 proto-oncogene. *J. Immunol.*, 158, 4584-4590.

Guarna, M.M., Lesnicki, G.J., Tam, B.M., Robinson, J., Radziminski, C.Z., Boraston, A., Jervis, E., Macgillivray, R.T.A. & Turner, R.F.B. (1997), On-line monitoring and control of methanol concentration in shake-flask cultures of *Pichia pastoris*. *Biotechnol. Bioeng.*, 56, 279-286.

Haaning, J., Oxvig, C., Overgaard, M.T. & Sottrupjensen, L. (1997), Simple and reliable procedure for PCR amplification of genomic DNA from yeast cells using short sequencing primers. *Biochem. Mol. Biol. Int.*, 42, 169-172.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Bendahman, N. & Hamers, R. (1993), Naturally occurring antibodies devoid of light chains. *Nature*, 363, 446-448.

Hollenberg, C.P. & Gellissen, G. (1997), Production of recombinant proteins by methylotrophic yeasts. *Curr. Opin. Biotechnol.*, 8, 554-560.

Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. & Oppermann, H. (1988), Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci.* USA, 85, 5879-5883.

Jimenez, E.R., Sanchez, K., Roca, H. & Delgado, J.M. (1997), Different methanol feeding strategies to recombinant *Pichia pastoris* cultures producing high level of dextranase. *Biotechnol. Tech.*, 11, 461-466.

Kalandadze, A., Galleno, M., Foncerrada, L., Strominger, J.L. & Wucherpfennig, K.W. (1996), Expression of recombinant HLA-DR2 molecules. Replacement of the hydrophobic transmembrane region by a leucine zipper dimerization motif allows the assembly and secretion of soluble DR alpha beta heterodimers. *J. Biol. Chem.*, 271, 20156-20162.

Koutz, P.J., Davis, G.R., Stillman, C.A., Barringer, K.J., Cregg, J.M. & Thill, G.P. (1989), Structural comparison of the *Pichia pastoris* alcohol oxidase genes. *Yeast*, 6, 167-177.

Linder, S., Schliwa, M. & Kube-Granderath, E. (1996), Direct PCR screening of *Pichia pastoris* clones. *Bio/Tech.*, 20, 980-982.

Liu, Z., Cashion, L.M. & Pu, H.F. (1998), Protein expression both in mammalina cell lines and in yeast *Pichia pastoris* using a single expression plasmid. *Bio/Tech.*, 24, 266-272.

Luo, D., Mah, N., Krantz, M., Wilde, K., Wishart, D., Zhang, Y. & Jacobs, F. (1995), Vl-linker-Vh orientation-dependent expression of single chain Fv-containing an engineered disulfide-stabilized bond in the framework regions. *J. Biochem.*, 118, 825-831.

Luo, D., Mah, N., Wishart, D., Zhang, Y., Jacobs, F. & Martin, L. (1996), Construction and expression of bifunctional proteins of single-chain Fv with effector domains. *J. Biochem.*, 120, 229-232.

Luo, D., Geng, M., Noujaim, A.A. & Madiyalakan, R. (1997a), An engineered bivalent single-chain antibody fragment that increases antigen binding activity. *J. Biochem.*, 121, 831-834.

Luo, D., Mah, N., Krantz, M., Wishart, D., Jacobs, F. & Martin, L. (1997b), High level secretion of single-chain antibody in *Pichia* expression system. *Biotechnol. Tech.*, 11, 759-761.

Martinezruiz, A., Delpozo, A.M., Lacadena, J., Mancheno, J.M., Onaderra, M. & Gavilanes, J.G. (1998), Secretion of recombinant pro- and mature fungal alpha-sarcin ribotoxin by the methyltropic yeast *Pichia pastoris* - the lys-arg motif is required for maturation. *Protein Expr. Purif.*, 12, 315-322.

McGrew, J.T., Leiske, D., Dell, B., Klinke, R., Krasts, D., Wee, S.F., Abbott, N. & Harrington, K. (1997), Expression of trimeric CD40 ligand in *Pichia pastoris*: use of a rapid method to detect high-level expressing transformants. *Gene*, 187, 193-200.

Ridder, R., Schmitz, R., Legay, F. & Gram, H. (1995), Generation of rabbit monoclonal antibody frag-

ments from a combinatorial phage display library and their production in the yeast. *Bio-Technol.*, 13, 255-260.

Scorer, C.A., Clare, J.J., McCombie, W.R., Romanos, M.A. & Sreekrishna, K. (1994), Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Bio-Technol.*, 12, 181-184.

Skerra, A. (1993), Bacterial expression of immunoglobulin fragments. *Curr. Opin. Immunol.*, 5, 256-262.

Sreekrishna, K., Brankamp, R.G., Kropp, K.E., Blankenship, D.T., Tsay, J.T., Smith, P.L., Wierschke, J.D., Subramaniam, A. & Birkenberger, L.A. (1997), Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene*, 190, 55-62.

Sudbery, P.E. (1996), The expression of recombinant proteins in yeasts. *Curr. Opin. Biotechnol.*, 7, 517-524.

White, C.E., Kempi, N.M. & Komives, E.A. (1994), Expression of highly disulfide-bonded proteins in *Pichia pastoris*. *Structure*, 2, 1003-1005.

White, C.E., Hunter, M.J., Meininger, D.P., White, L.R. & Komives, E.A. (1995), Large-scale expression, purification and characterization of small fragments of thrombomodulin - the roles of the 6th-domain and of methionine-388. *Protein Eng.*, 8, 1177-1187.

Wung, J.L. & Gascoigne, N.R.J. (1996), Antibody screening for secreted proteins expressed in *Pichia pastoris*. *BioTech*, 21, 808-811.

Yokota, T., Milenic, D.E., Whitlow, M. & Schlom, J. (1992), Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.*, 52, 3402-3408.

Production of antibodies in transgenic plants

J.W. Larrick, L. Yu, J. Chen, S. Jaiswal and K. Wycoff

Palo Alto Institute of Molecular Medicine, 2462 Wyandotte Street, and Planet Biotechnology Inc., 2438 Wyandotte Street, Mountain View, CA 94043 (USA)

A) Plant bioreactors

The first transgenic plants were reported in 1983 (Fraley *et al.*, 1983; Zambryskyi *et al.*, 1983). Since then, many recombinant proteins have been expressed in several important agronomic species of plants including tobacco, corn, tomato, potato, banana, alfalfa (Austin *et al.*, 1994) and canola (summarized in Kusnadi *et al.*, 1997a). Recent work suggests that plants will be a facile and economic bioreactor for large-scale production of industrial and pharmaceutical recombinant proteins (Kusnadi *et al.*, 1997b; Austin *et al.*, 1994; Krebbers *et al.*, 1992; Whitelam *et al.*, 1993). Genetically engineered (transgenic) plants have several advantages as sources of proteins compared with human or animal fluids/tissues, recombinant microbes, transfected animal cell lines or transgenic animals. These include:

(1) production of raw material on an agricultural scale at low cost;

(2) efficiency of the transformation technology and speed of scale-up;

(3) correct assembly of multimeric antibodies (unlike bacteria);

(4) increased safety, as plants do not serve as hosts for human pathogens, such as HIV, prions, hepatitis viruses, etc.;

(5) reduced capitalization costs relative to fermentation methods.

Perhaps most important are the cost benefits of plant production. For example, Kusnadi *et al.* (1997b) calculated the cost of producing a recombinant protein in various agricultural crops (see fig. 1). The cost estimate was based on the commodity price of the crop, the fraction of total protein in the crop, and the not unreasonable assumption that the recombinant protein accumulated to 10% of the total plant protein. Although crops with more protein content (e.g. soybeans, 40% versus potatoes 2%) are more

Received July 10, 1998.

10. The STE solution used to render the bacterial periplasm hypertonic typically contains little rFab after the bacteria are pelleted. However, rFab leakage may occur if the bacteria are incubated in STE for more than 1 h. Therefore, do not incubate the bacteria in STE for more than 45 min on ice.
11. After centrifugation, care should be taken not to pour the cells out of the bottle because the pellet is loose after incubation in STE. It may be necessary to respin the bottles to form appropriate conditions for good pellet formation.
12. By comparing Western blotting results of crude rFab periplasmic extracts, we observed that the total amount of rFab reactive to anti-mouse Fab Ab was not always detected by the Ni-NTA conjugate. This suggests that the His tag fused to the HC can be proteolytically cleaved within the bacterial periplasm. Therefore, the successful purification of rFabs should be checked after metal chelate chromatography and before further purification. The addition of protease inhibitors to the periplasmic extract may help reduce proteolysis.
13. Concentrated rFab preparations may be subjected to size-exclusion chromatography to remove residual impurities, such as aggregated rFabs and contaminating *E. coli* proteins, which can co-elute from the His-bind column. This may increase the avidity of the rFab preparation.

References

1. Barbas, C. F., Kang, A. S., Lerner, R. A., and Benkovic, S. J. (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc. Natl. Acad. Sci. USA* **88**, 7978–7982.
2. Raffal, R., Vukmirica, J., Weisgraber, K. H., Rassart, E., Innerarity, T. L., and Milne, R. (1999) Bacterial expression and purification of the Fab fragment of a monoclonal antibody specific for the low density lipoprotein receptor-binding site of human apolipoprotein E. *Protein Exp. Purif.* **16**, 84–90.
3. Huse, W. D., Sastry, L., Iverson, S. A., Kang, A. S., Alting-Mees, M., Burton, D. R., Benkovic, S. J., and Lerner, R. A. (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* **246**, 1275–1281.
4. Bothmann, H. and Plückthun, A. (1998) Selection for a periplasmic factor improving phage display and functional periplasmic expression. *Nature Biotechnol.* **16**, 376–380.
5. Kipriyanov, S. M., Moldenhauer, G., and Little, M. (1997) High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures. *J. Immunol. Methods* **200**, 69–77.
6. Raffal, R., Weisgraber, K. H., MacKenzie, R., Rupp, B., Rassart, E., Hirama, T., Innerarity, T. L., and Milne, R. (2000) Binding of an antibody mimetic of the human low density lipoprotein receptor to apolipoprotein E is governed through electrostatic forces. *J. Biol. Chem.* **275**, 7109–7116.
7. Forsberg, G., Forsgren, M., Jaki, M., Norin, M., Sterky, C., Enhörning, Å., et al. (1997) Identification of framework residues in a secreted recombinant antibody fragment that control production level and localization in *Escherichia coli*. *J. Biol. Chem.* **272**, 12,430–12,436

31

Expression of Antibody Fragments in *Pichia pastoris*

Philipp Holliger

1. Introduction

Since the advent of hybridoma technology 25 years ago, monoclonal antibodies (Abs) have revolutionized many aspects of biological research and health care. After some initial setbacks, Abs are also beginning to make an impact as therapeutic agents in the clinic *(1)*. In the last decade, novel selection technologies, such as phage display and ribosome display, have emerged, allowing the isolation of Abs directly from diverse repertoires of V genes *(2)*. Phage display, in particular, has become a mature technology, allowing Abs with nanomolar (or even subnanomolar) affinities to be made to order against virtually any Ag, including self Ags (*3*; *see* Note 1). Furthermore, using high-throughput technologies, such as robotics and array screening, a multitude of Abs against a given Ag (or mixtures thereof) can now be isolated simultaneously, greatly increasing the options for assay or drug development (*see* Note 2).

Regardless of the method of isolation, Abs have to be expressed in recombinant form for screening, characterization, and application. Although both whole Abs and Ab fragments (Fabs *[4]*, Fvs *[5]*, scFvs *[6]*, and diabodies *[7]*) can be expressed in eukaryotic cells (e.g., mammalian yeast, plant, and insect cells *[8]*), it is time-consuming and cost-intensive. Expression in bacteria, particularly secretion to the bacterial periplasm (*see* Note 3), is a quick and cheap alternative and is best-suited for the screening and characterization of a large number of Ab variants. Some phagemid vectors *(9)* even offer a built-in switch between phage display and soluble expression, allowing direct screening of Ab fragments isolated by phage selection without the need for

From: *Methods in Molecular Biology, vol. 178: Antibody Phage Display: Methods and Protocols*
Edited by: P. M. O'Brien and R. Aitken © Humana Press Inc., Totowa, NJ

Appendix IV
UCAL-269
09/425,075

recloning. However, because of the lack of glycosylation, only Ab fragments and not whole Abs (*see* above) can be produced in a functional form in bacteria.

Expression yields in bacteria can vary widely between different Ab fragments, but yields of 1–10 mg/L are typical for shaker-flask cultures. Using fermentation technology, expression levels of up to 1 g/L can be reached *(10)*. Generally, expression yields are a function of the Ab fragment sequence and format (e.g., Fv vs Fab), rather than the expression system. Fvs (5–50 mg/L) often give the highest expression yields (but are sometimes unstable), followed by scFvs, then diabodies, with Fabs usually giving the lowest expression yields (0.1–1 mg/L). As a rule of thumb, Ab fragments derived from phage libraries tend to give higher yields than those recloned from hybridomas. However, some Ab fragments are generally difficult to express in *Escherichia coli*. Although yields of difficult fragments can sometimes be improved through protein engineering *(11,12)* or selection *(12)*, no general rules have emerged.

A pragmatic alternative to time-intensive optimization of bacterial expression is the use of a eukaryotic expression host. The methylotrophic yeast, *Pichia pastoris*, combines some of the advantages of eukaryotic expression systems, e.g., more efficient folding of multidomain and cys-rich proteins, with the speed and cost efficiency approaching that of prokaryotic systems *(13)*. Optimal expression in *Pichia* is dependent on a range of factors, including codon usage *(14)*, aeration, temperature control (at 28–30°C: *Pichia* is temperature-sensitive) and methanol (MeOH) concentration (when using the alcohol oxidase 1 [AOX1] promoter). Protease-sensitive proteins are usually not well expressed, because *Pichia* secretes a number of proteases. Nevertheless, *Pichia* has become a popular host for heterologous protein expression *(13)*, and a range of Ab fragments, including scFvs and diabodies, have been successfully expressed in *Pichia*, with yields up to 200 mg/L *(15)* in shaker flasks and >1 g/L in fermentor cultures.

This chapter focuses on the expression of functional Ab fragments by the yeast, *P. pastoris (3)*. Using appropriate expression vectors, the Abs are secreted into the yeast culture supernatant, and purified using affinity chromatography. The Ag specificity and binding affinity of the Abs can be determined using BIAcore technology or other suitable methods.

2. Materials

1. *P. pastoris* strain, GS115 (Invitrogen) (*see* Note 4).
2. YP medium: 1% (w/v) yeast extract, 2% peptone.
3. YPD medium: 1% yeast extract, 2% peptone, 2% glucose.
4. YPDS medium: 1% yeast extract, 2% peptone, 2% glucose, 1 *M* sorbitol. For YPDS plates, add 2% (w/v) agar.

5. Sterile Millipore H_2O.
6. 1 *M* sorbitol (SORB).
7. *Pichia* expression vectors: pPIC (AOX promoter) or pGAPZ (Invitrogen) (*see* Note 4). Both vectors have a C-terminal c-myc epitope tag for convenient immunodetection with an anti-myc Ab (9E10) (Invitrogen), as well as a C-terminal hexahistidine tag for immobilized metal-affinity chromatography (IMAC) purification.
8. Ab clone. Because bicistronic expression works only poorly in *Pichia* (unlike *Escherichia coli*), it is preferable to use single-chain Ab formats (e.g., scFv, diabody). Two-chain Ab formats (e.g., Fvs, Fabs, bispecific diabodies) require that the two chains be cloned and transformed separately.
9. Appropriate restriction enzymes and DNA purification and other reagents for molecular cloning of Ab sequences.
10. *E. coli* strain for propagation of plasmid vectors, e.g., TG1.
11. Zeocin (Invitrogen): stock solution 100 mg/mL. Store at –20°C (in the dark).
12. 2TY medium, supplemented with 0.1–5% (w/v) glucose. Autoclave for sterilization, then supplement with sterile-filtered (0.2 µm) glucose (20%).
13. TYE agar (for plates), supplemented with 0.1–5% (w/v) glucose. Autoclave, then supplement with sterile-filtered glucose (20%).
14. TE: 10 m*M* Tris-HCl, 1 m*M* ethylyenediamine tetraacetic acid (EDTA), pH 8.0. Filter-sterilize.
15. Electroporator, e.g., Bio-Rad Genepulser.
16. Methanol.
17. 1 *M* Phosphate buffer: 132 mL 1 *M* KH_2HPO_4, 868 mL 1 *M* KH_2PO_4. Adjust pH to 6.0 with KOH. Filter-sterilize.
18. 10X YNB: 134 g yeast nitrogen base (with NH_4SO_4)/L MilliQ H_2O. Autoclave.
19. 500X B: 20 mg biotin/100 mL MilliQ H_2O. Filter-sterilize.
20. 10X GY: 10% glycerol (v/v) in MilliQ H_2O. Filter-sterilize.
21. BMGY: 100 mL 1 *M* phosphate buffer, pH 6.0, 100 mL 10X YNB, 2 mL 500X B, 100 mL 10X GY in 1 L of YP medium. Filter-sterilize.
22. BMMY: as BMGY, but replace the 10X GY with 100 mL 5% MeOH (v/v) in H_2O. Filter-sterilize.
23. BIAcore machine and software, CM5 BIAcore chip.
24. N-ethyl-Ni-(diaminopropyl) carbodimide (EDC); (*N*-hydroxysuccimide (NHS).
25. Ag of interest, purified.
26. 100 m*M* Na acetate, pH 6.0–4.0; 1 *M* ethanolamine.
27. Phosphate buffered saline (PBS).
28. Ni-NTA resin (Qiagen).
29. IMAC phosphate buffer: 29.82 g NaH_2PO_4, 5.52 g $NaH_2PO_4 \cdot H_2O$, 147 g NaCl/L. Adjust the pH to 7.5 with 1 *M* NaOH.
30. Imidazole (Sigma).
31. IMAC Loading buffer: 50 mM IMAC phosphate buffer, pH 7.5, 0.5 *M* NaCl, 20 m*M* imidazole. Dilute IMAC phosphate buffer fivefold in H_2O, then add imidazole powder to give a final concentration of 20 m*M*. Store at 4°C.

3. Methods

3.1. Preparation of Electrocompetent Pichia GS115

1. Inoculate a single colony of *Pichia* GS115 into 5 mL YPD medium and grow overnight at 30°C.
2. Dilute the overnight culture 1:1000 into fresh YPD medium (e.g., add 1 mL overnight culture into 1 L) and grow overnight at 30°C.
3. Pellet the cells at 1500*g* for 20 min at 4°C, then resuspend in an equal volume of ice-cold sterile Millipore H_2O.
4. Pellet the cells, then resuspend in 0.5 vol ice-cold Millipore H_2O.
5. Pellet the cells, then resuspend in 0.2 vol ice-cold sterile 1 *M* SORB.
6. Pellet the cells, then resuspend in 0.005 vol ice-cold sterile 1 *M* SORB.
7. Use the cells for transformation, or store in 0.1 mL aliquots by flash-freezing on dry ice and store at –70°C (*see* Note 5).

3.2. Cloning of Ab Fragments for Expression in P. pastoris

1. Clone the selected Ab fragment(s) into the appropriate *Pichia* expression vector in *E. coli* using standard cloning procedures (*see* Notes 6 and 7).
2. Prepare plasmid DNA from the resulting clones by miniprep procedures, then linearize with *Avr*II (pGAPZα) or *Bst*XI (pPICZ). Extract the digests with phenol:chloroform (1:1) once, and precipitate the DNA with ethanol. Resuspend the precipitated pellet in 5 µL TE.
3. Add 2.5 µL DNA to 50 µL electrocompetent *Pichia* cells and electroporate at 1.5 kV, 25 µF, and 200 Ω. Resuspend the cells in 1 mL 1 *M* SORB and incubate for 2 h at 30°C.
4. Plate the transformed cells on YPDS plates containing 50 µg/mL zeocin and incubate at 30°C. Colonies (10–1000) will appear in 3–4 d.

3.3. Expression of Ab Fragments in Pichia (see Note 8)

3.3.1. Expression in pPIC (MeOH Induction) (see Note 9)

1. Inoculate a colony expressing a pPIC/Ab clone into 1 mL YPD medium containing 0.1mg/mL zeocin and grow overnight at 30°C.
2. Dilute the overnight culture 1:100 into fresh YP medium (e.g., dilute 0.1 mL into 10 mL) and grow for 24 h at 30°C. Add MeOH to a final concentration of 0.5% (v/v) and grow for a further 24 h. Repeat the MeOH addition every 24 h until the culture is harvested after 1–4 d (*see* Note 10).

   Alternatively, dilute the overnight culture 1:100 into fresh BMGY medium (e.g., dilute 0.1 mL into 10 mL) and grow at 30°C to an optical density 600 nm of 4.0. Pellet the cells by centrifugation at 1500*g* for 20 min, then resuspend in an equal volume of BMMY medium and grow for 24 h at 30°C. Add MeOH (0.5% [v/v] final concentration) and grow for a further 24 h. Repeat the MeOH addition every 24 h until the culture is harvested (after 1–4 d).
3. Spin the culture at 10,000*g* for 30 min at 4°C and collect the supernatant (*see* Note 11). The supernatant can be used directly for analysis of Ab expression (e.g. by enzyme-linked immunosorbant assay [ELISA] or BIAcore) or can be stored and/or purified before use (*see* Notes 12 and 13).

3.3.2. Expression in pGAPZ (Constitutive Expression)

1. Inoculate a colony expressing a pGAPZα/Ab clone into 1 mL YPD medium containing 0.1 mg/mL zeocin and grow overnight at 30°C.
2. Dilute the overnight culture 1:100 (e.g., dilute 0.1 mL into 10 mL) into fresh YPD medium (without zeocin) (*see* Note 14). Grow the culture at 30°C for 1–4 d (*see* Note 11).
3. Harvest the culture supernatant and store or purify the Ab as described in Subheading 3.3.1.

3.4. Analysis of Ab Binding by BIAcore (see Note 15)

This procedure can be used to quickly investigate Ab specificity using crude extracts of yeast culture supernatant as an alternative to ELISA (*see* Note 16). If purified material is used, the method can also be used to determine affinity. More information about the BIAcore instrument and the method can be found at the BIAcore website: http://www.biacore.com.

1. Dock a research-grade CM5 chip (BIAcore) in the BIAcore machine, according to the manufacturer's instructions.
2. Amine-couple 500–5000 resonance units (RU) of the desired Ag, according to the manufacturer's instructions (the amount of Ag this corresponds to depends on its molecular weight, because the BIAcore signal [RU] is mass-dependent). Briefly, activate the chip surface with EDC–NHS (typical injection is 30 µL at 10 µL/min flow rate). Inject the Ag (typically, 100 µg/mL in 100 m*M* Na acetate, pH 6.0–4.0) (*see* Note 17). Stop the coupling reaction by injecting 1.0 *M* ethanolamine, which blocks the remaining activated sites.
3. Filter the recombinant Ab samples through a 0.2 µm filter before injection.
4. Pass the Ab solution over the chip surface (typical injection times range from 1 to 10 min at flow rates of 5–50 µL/min). An increase in RU indicates binding.
5. Plot a graph of RU vs time. Analyze the binding affinity and/or kinetics using the BIAcore software.

3.5. Purification of Recombinant Ab Fragments by IMAC

Like Ab fragments expressed from polyhistidine-tagged *E. coli* expression vectors, Abs expressed in *P. pastoris* using the pPIC or pGAPZ plasmids can be purified by IMAC. The Ab-containing culture supernatants must first be dialyzed against PBS before purification to remove chelating compounds present in the growth media (*see* Note 18).

1. Dialyze the culture supernatant against two changes of PBS (ideally at 4°C). For smaller volumes, dialysis tubing with a 10 kDa cutoff is suitable. For large

volumes, dialysis is best performed using tangential flow filtration using repeated addition of PBS during the concentration process (*see* Note 12).

2. Add the appropriate amount of Ni-NTA resin to an appropriate column and equilibrate the resin with 10 column volume loading buffer (e.g., for 5 mL resin, use 50 mL buffer). 1 mL Ni-NTA resin is usually sufficient to purify 2–3 mg Ab fragment (*see* Note 19).
3. Load the dialyzed Ab preparation onto the column (either by gravity flow or using a peristaltic pump) and collect the unbound fraction.
4. Wash the column with at least 10 column volume loading buffer. If the washing process can be observed using an UV-flowcell, washing should continue until a stable baseline is reached.
5. Elute the Ab fragments using an imidazole gradient from 35 to 200 m*M* in loading buffer (*see* Note 20). Elution peak fractions should ideally be detected using an UV-flowcell. The elution of Ab should be confirmed by ELISA or BCA protein assay (Pierce).
6. Dialyze the Ab fractions into the desired buffer (e.g., PBS) to remove the imidazole, then concentrate the Ab by ultrafiltration using a stirred cell device with an appropriate cutoff (10 kDa for scFvs, 30 kDa for Fabs, and diabodies).
7. Aliquot the Ab preparations for storage. Concentrated Ab preparations (>0.5 mg/mL) in PBS are suitable for freezing. As a rule, preparations should always be flash-frozen in dry ice or liquid nitrogen and never in a –20°C freezer. Once frozen, a –20°C freezer is suitable for short- to medium-term storage.

4. Notes

1. Protocols for the selection of Ab specificities from phage libraries have been published *(16)* and several libraries are available to researchers (http://www.mrc-cpe.cam.ac.uk/phage/index.html).
2. An alternative method for isolating human Abs is transgenic "human" mice with partial human heavy- and light-chain loci inserted into their genomes *(17)*. A possible advantage of this approach may be the ability to use the isolated hybridomas directly for production of whole Abs with no need for further genetic manipulation.
3. Ab fragments can be expressed both intra- and extracellularly, i.e., secreted. Intracellular expression of Ab fragments in *E. coli* usually gives rise to insoluble aggregates (inclusion bodies) that have to be refolded. Secretion from bacteria (to the periplasm) or yeast mimics the natural expression and folding pathway of Abs and often provides a more direct route to functional Ab fragments.
4. *P. pastoris* strains and expression vectors are commercially available from Invitrogen. *Pichia* protocols are available to download from the Invitrogen website (http://www.invitrogen.com/manuals.html).
5. Freezing reduces competence. In order to obtain the highest possible transformation efficiencies, it is advisable to use freshly prepared cells. However, frozen competent cells are perfectly adequate for standard transformations. Before use, thawed frozen cells should be washed once in 0.5 mL ice-cold sterile 1 *M* sorbitol.
6. Ab fragments can be cloned using PCR directly from hybridomas using standard methodology (a kit comprising mouse V-gene-specific primers is available from Pharmacia) or isolated from phage selected from libraries using panning procedures.
7. When using zeocin selection in combination with high-salt media (TYE, 2TY), it is advisable to use a final concentration of 100 µg/mL zeocin for selection. Transformed *E. coli* cells should be incubated for 1–2 h in 2TY, 1% glucose at 37°C, before plating on zeocin plates because zeocin resistance is expressed slowly.
8. There are two types of promoter systems available in *Pichia*; the MeOH-inducible AOX1 promoter and the constitutive glyceraldehyde-phosphate dehydrogenase (GAPDH) promoter. Expression of some proteins can be higher under control of the GAPDH promoter (using glucose as a carbon source) than by MeOH induction of the AOX1 promoter. Both promoters should be tried because expression yields can differ dramatically. Furthermore, expression levels usually vary a great deal among different *Pichia* clones. It is advisable to screen a number of colonies for expression in order to identify high-expressing "jackpot" clones. *Pichia* expression can also depend on good aeration so expression cultures should be grown with vigorous shaking (350 rpm).
9. For optimal protein yields with MeOH induction, the alternative may be more effective than the primary methods.
10. Protein expression takes place over 1–4 d at 30°C. Maximum yields usually are obtained by harvesting on d 2 or 3.
11. Respin the culture if the supernatant is not clear.
12. The cleared supernatant can be used directly in ELISA or BIAcore analysis, or can be stored at –20°C prior to purification. For large-scale preparations (>1 L), it may be advantageous to concentrate the supernatant before purification. Various concentration methods are available (e.g., ammonium sulphate precipitation), but ultrafiltration is preferable. Filter the supernatant through a 16 µm tangential-flow filter (Flowgen Minisette system) with the use of a peristaltic pump to remove small debris. Concentrate the supernatant using the Minisette system, using a tangential-flow filter minisette with an appropriate cutoff (e.g., 10 kDa for scFvs and Fvs, or 30 kDa cutoff for Fabs and diabodies). The concentrate (typically, 0.3–0.5 L) can be stored at –20°C prior to purification.
13. Ab fragments produced in *Pichia* often have nonhomogenous N-termini because of incomplete processing of the leader peptide, giving rise to fuzzy bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. These N-terminal extensions can be shaved off using 5 µg/mL trypsin digestion for 5 min (immobilized TPCK trypsin [Pierce]). The reaction is stopped by addition of Pefabloc trypsin inhibitor (at 5 µg/mL) and removal of the enzyme gel by centrifugation. Because trypsin may also cleave off polyhistidine tags, it is advisable to carry out the digestion after purification.

14. Zeocin selection during expression is unnecessary and can reduce the yield of expressed protein.
15. These methods are not limited to Ab expressed in *Pichia* and can also be used for determining Ag specificity (crude periplasmic extracts) and binding affinity (purified Ab) of Ab expressed in *E. coli*.
16. Either ELISA or BIAcore can be used to determine affinity constants of purified Ab fragments. In my opinion, BIAcore is superior to ELISA-based methods, provided attention is paid to the oligomerization state of the Ab fragment. Multimeric fragments (e.g., some scFvs, bivalent diabodies) bind to solid-phase Ags with much-increased affinity (avidity). Failure to take this into account can lead to an overestimation of affinity by several orders of magnitude. On the other hand, multimerization can be helpful in increasing the sensitivity of Ag-binding assays, particularly for Ab fragments with modest affinities for Ag. For methods relating to Ab multimerization (and expression), *see* ref. *18*. BIAcore can also be used to measure Ag-binding kinetics.
17. For optimal coupling efficiencies, the pH should be determined by experimental analysis (knowledge of the isoelectric point value of the Ag is not sufficient). Coupling should be spontaneous. For slow-reacting Ags, it may be appropriate to slow down the flow rate.
18. Purification by IMAC has advantages beyond other purification methods because of its versatility and mild elution conditions. The commonly used rich medium for *Pichia* (YP) expression (and for *E. coli* [2TY or Luria-Bertani broth]) contain metal-chelating compounds, which strip the metal from the IMAC column (the same also applies for periplasmic preparations from *E. coli*-containing EDTA). Metal loss from the IMAC column is easy to spot because the column loses its blue-green color (in the case of Ni^{2+}) and turns white.
19. Ab fragments can give widely differing expression yields, ranging from 1 to 100 mg/L of induced *Pichia* culture. It is thus advisable to determine approximate expression levels before embarking on purification.
20. Most Ab fragments elute between 50 and 100 m*M* imidazole. Diabodies and triabodies, which have two and three hexahistidine tags, respectively, usually elute at higher concentrations (50–200 m*M* imidazole).

References

1. Milstein, C. and Waldmann, H. (1999) Optimism after much pessimism: what next? *Curr. Opin. Immunol.* **11**, 589–591.
2. Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994) Making antibodies by phage display technology. *Ann. Rev. Immunol.* **12**, 433–455.
3. Griffiths, A. D. and Duncan, A. R. (1998) Strategies for selection of antibodies by phage display. *Curr. Opin. Biotechnol.* **9**, 102–108.
4. Better, M., Chang, C. P., Robinson, R. R., and Horwitz, A. H. (1988) *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* **240**, 1041–1043.
5. Skerra, A. and Pluckthun, A. (1988) Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* **240**, 1038–1041.

6. Huston, J. S., Levinson, D., Mudgett, H. M., Tai, M. S., Novotny, J., Margolies, M. N., et al. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **85**, 5879–5883.
7. Holliger, P., Prospero, T., and Winter, G. (1993) "Diabodies": small bivalent and bispecific antibody fragments. *Proc. Natl Acad. Sci. USA* **90**, 6444–6448.
8. Verma, R., Boleti, E., and George, A. J. (1998) Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems. *J. Immunol. Methods* **216**, 165–181.
9. Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., and Winter, G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res.* **19**, 4133–4137.
10. Carter, P., Kelley, R. F., Rodrigues, M. L., Snedecor, B., Covarrubias, M., Velligan, M. D., et al. (1992) High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Biotechnology* **10**, 163–167.
11. Knappik, A. and Pluckthun, A. (1995) Engineered turns of a recombinant antibody improve its in vivo folding. *Protein Engineering* **8**, 81–89.
12. Coia, G., Ayres, A., Lilley, G. G., Hudson, P. J., and Irving, R. A. (1997) Use of mutator cells as a means for increasing production levels of a recombinant antibody directed against hepatitis B. *Gene* **201**, 203–209.
13. Hollenberg, C. P. and Gelissen, G. (1997) Production of recombinant proteins by methylotrophic yeasts. *Curr. Opin. Biotechnol.* **8**, 554–560.
14. Sreekrishna, K., Brankamp, R. G., Kropp, K. E., Blankenship, D. T., Tsay, J. T., Smith, P. L., et al. (1997) Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* **190**, 55–62.
15. Luo, D., Mah, N., Krantz, M., Wishart, D., Jacobs, F., and Martin, L. (1997) High-level secretion of single-chain antibody in *Pichia* expression system. *Biotechnol. Tech.* **11**, 759–761.
16. Harrison, J. L., Williams, S. C., Winter, G., and Nissim, A. (1996) Screening of phage antibody libraries. *Methods Enzymol.* **267**, 83–109.
17. Bruggeman, M. and Taussig, M. J. (1997) Production of human antibody repertoires in transgenic mice. *Curr. Opin. Biotechnol.* **8**, 503–508.
18. Tomlinson, I. and Holliger, P. (2000) Methods for generating multivalent and bispecific antibody fragments. *Methods Enzymol.* **326**, 461–479.